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Brief Articles

Bone Marrow Mesenchymal Stromal Cells from Patients with Acute and Chronic Graft-versus-Host Disease Deploy Normal Phenotype, Differentiation Plasticity, and Immune-Suppressive Activity

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The success of allogeneic hematopoietic stem cell transplantation (allo-HSCT) is often limited by the development of acute and/or chronic graft-versus-host disease (GVHD). The lack of effective therapies to treat steroid-refractory GVHD patients has bolstered clinical evaluation of mesenchymal stromal cell (MSC) therapy for GVHD. Currently, testing of MSCs for the treatment of GVHD has exclusively used allogeneic MSCs despite emerging evidence that MSCs lose their immunoprivileged status in vivo. We hypothesized that autologous MSCs could be a viable alternative MSC source for treating active GVHD. MSCs were isolated and successfully expanded from the bone marrow of 12 volunteers (ages 2 to 55 years) who had allo-HSCT transplants and subsequently developed GVHD. MSCs from subjects with GVHD demonstrated an initial lag in growth compared with healthy control subjects; however, this lag disappeared with continued ex vivo expansion. Immunophenotype and mesodermal differentiation capacity of MSCs from GVHD patients were indistinguishable from that of healthy control MSCs. In vitro immunomodulatory functional analyses also demonstrated that GVHD MSCs were equivalent to healthy control MSCs with regards to dose dependently suppressing T cell proliferation and up-regulating indoleamine 2,3-dioxygenase expression when primed with IFN-γ. Single tandem repeat chimerism analyses further demonstrated that MSCs expanded from GVHD patients were exclusively recipient derived. Based on these data, we conclude that recipient-derived MSCs from patients with GVHD are analogous to MSCs from healthy volunteers and represent a viable option for clinical testing as an immunomodulatory option for symptomatic GVHD.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective treatment for aggressive leukemia and often represents the only option for cure. Alas, a portion of allo-HSCT subjects develop graft-versus-host disease (GVHD) as a serious life-threatening side effect. Attempts to bolster acute and chronic GVHD therapy with immunosuppressants, monoclonal antibody therapy, and photopheresis have so far proven ineffective, because of lack of efficacy, increased incidence of infection, and increased toxicity [1-6]. Understandably, novel means to mitigate the effects of GVHD are a priority.

Mesenchymal stromal cells (MSCs) have been regarded as a promising cellular therapeutic for the treatment of GVHD because of their anti-inflammatory and tolerogenic properties [7]. Despite encouraging phase I and phase II trials involving allogeneic MSCs for GVHD [8-13], clinical efficacy has not been definitely demonstrated [14]. One possibility for the lack of efficacy may hinge on the premise that allogeneic MSCs are immune privileged and can therefore be administered universally to any patient. Our research and that of others have clearly demonstrated that this "universal donor"

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premise is flawed because allogeneic MSCs are immune rejected in vivo through both innate and adaptive immune responses [15-17]. Thus, allogeneic MSCs may not be well suited for immune-mediated disorders, like GVHD, which may require multiple infusions to obtain a durable therapeutic response.

Whether recipient-derived MSCs for the treatment of GVHD could be a viable option is unclear; however, in 1995 Lazarus et al. [18] demonstrated that despite high-dose chemotherapy and radiation, MSCs could be isolated from some patients who had undergone HSCT for hematologic malignancies. Investigators have since shown that MSCs can be isolated from patients with active acute lymphoblastic leukemia [19], acute myelogenous leukemia [20], and multiple myeloma [21]; however, no study has evaluated whether MSCs can be isolated from subjects with pre-existing or evolving GVHD. In this study we obtained bone marrow from allo-HSCT volunteers with active GVHD, determined feasibility of isolating and expanding their MSCs, and then ascertained the clinical utility of their MSCs by comparing immunophenotype, plasticity and immune-modulating functions to MSCs from healthy volunteers.

METHODS

MSC Isolation and Culture

After institutional review board approval and informed consent, MSCs were isolated from 10 to 20 mL of bone marrow from the iliac crest of healthy volunteers and subjects with GVHD. Bone marrow aspirates were diluted 1:2 with PBS and layered onto a Ficoll density gradient. The cells were centrifuged at 400 $\times g$ for 20 minutes with no brake and the mononuclear cells collected, washed, and plated in complete human MSC medium (α-MEM, 10% human platelet lysate [hPL], or 10% FBS, 100 U/mL penicillin/streptomycin) at 100,000 to 300,000 cell/cm^2. Nonadherent hematopoietic cells were removed by changing the medium after 3 days of culture, and MSCs were allowed to expand for 7 to 12 days. Thereafter, the cells were passaged weekly by treatment with trypsin/EDTA and reseeded in fresh MSC medium at 1000 cells/cm^2.

Characterization of Bone Marrow Aspirates

A 100-μL sample of undiluted marrow was analyzed using a particle analyzer (BD Coulter, Indianapolis, IN) to generate a WBC differential.

Preparation of Platelet Lysate

Outdated platelet pheresis products were purchased from the American Red Cross, which meet all American Association of Blood Banks (AABB) and US Food and Drug Administration regulatory requirements for sterility and infectious disease screening for transfusion products. To generate pooled hPL (phPL), we used a freeze–thaw procedure to fracture the platelets followed by a fibrinogen depletion procedure, previously described [22].

MSC Cell Expansion

MSCs were plated at 1000 cell/cm^2 and cultured for 5 to 7 days in either FBS or phPL media and then counted using a Countess automated cell counter (Invitrogen, Grand Island, NY). Cell sizes were also determined were using both the Countess automated cell counter and by flow cytometry using forward and side scatter measurements.

MSC Differentiation

At passage 3, MSCs from normal and GVHD subjects were seeded at 3000 cells/cm^2 for adipocyte and osteoblastic differentiation for 3 weeks. Adipogeneic media consisted of complete culture medium supplemented with .5 mM dexamethasone, .5 μM isobutylmethylxanthine, and 50 μM indomethacin, whereas osteoblastic media consisted of complete culture medium supplemented with 1 nM dexamethasone, 20 mM β-glycerol phosphate, and 50 μM L-ascorbic acid-2-phosphate. After 3 weeks of differentiation MSCs were washed, fixed with 2% formalin/PBS for 1 hour, and then stained with either Alzarin Red to show osteoblastic differentiation or Oil Red to show adipogenic differentiation.

Immunophenotyping MSC by Flow Cytometry

MSCs were cultured for 7 days in phPL media, harvested and resuspended at a concentration of 1 $\times$ 10^6 cells/mL, and analyzed by flow cytometry for the expression of CD45, CD34, CD44, CD73, CD90, CD105, and HLA-I (BD BioSciences). All samples were run on a Canto II flow cytometry (Beckman Coulter, Indianapolis, IN) with the appropriate isotype control subjects. Data are presented as mean fluorescent intensity difference compared with isotype control subjects.

Real-Time Quantitative PCR

MSCs were cultured for a minimum of 7 days and primed for 4 hours with 10 ng/mL recombinant human IFN-γ. DNA-free total RNA was extracted and reverse transcribed as described [23]. Real-time quantitative PCR assays were performed in duplicate on an ABI 7500 Fast Real-Time PCR system thermal cycler and SYBR Green Mastermix (Applied Biosystems, Grand Island, NY) with human primer sequences for indoleamine 2,3-dioxygenase (IDO) and β-actin. Primers were designed using the NCIB/Primer Blast designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Data were analyzed using the relative quantification method [24].

T Cell Proliferation Assay

Blood was obtained from healthy volunteers after informed consent on an institutional review board–approved protocol. Human peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll density gradient. PBMCs were cultured at 400,000 cells per well in a 24-well plate with or without MSCs in 10% FBS RPMI. T lymphocytes were stimulated using anti-CD3/anti-CD28 Dyna beads (Invitrogen). T cell proliferation was determined 4 days later by flow cytometry analysis of Ki67 expression.

Single Tandem Repeat Chimerism Analysis

Single tandem repeat (STR) chimerism testing is used to detect and quantify donor blood cells present in recipient bone marrow or peripheral blood post-transplant allo-transplant. At the Emory University Hospital HLA laboratory, 15 autosomal STR locus-specific primer sets and the Amelogenin gender determining marker are used to determine STR chimerism [25]. The percent engraftment is determined by the area under the curves of peaks generated from the detectable emission energy of fluorophores (eg, 6-FAM, VIC, NED, PET, and LIZ) and the number of molecules present.

DNA was extracted from unfractionated whole blood obtained pre-transplant from each recipient and his or her paired donor. DNA was also extracted from purified MSCs isolated from each recipient post-transplant and cultured in vitro. The extracted DNA was diluted to a final concentration of .125 ng/μL Fifteen autosomal STR markers (including 13 CODIS core loci, D19S433, and D2S1338) were typed along with Amelogenin using the AmpFeSTR Identifiler Plus kit (Promega Corporation, Madison, WI). PCR amplification was carried out using an MJ research PTC-100 themocycler (Perkin Elmer, Santa Clara, CA), according to kit protocols. After PCR, 1 μL of the amplified products were diluted into 8.7 μL of Hi–Di formamide (Applied Biosystems) and .3 μL of LIZ GS500 internal size standard (Applied Biosystems). Samples were electrokinetically injected at 3 kV for 10 seconds and separated on a 3130xl Genetic Analyzer (Applied Biosystems) using POP-4 polymer (Applied Biosystems) on a 36-cm capillary array (Applied Biosystems). Genotyping was then performed in GeneMapperID v3.2 (Applied Biosystems) using manufacturer provided bins and panels.

Statistical Analysis

Data are reported as mean ± SD. All calculations were carried out using GraphPad Prism software (GraphPad Software, La Jolla, CA). Comparisons between groups were made by analysis of variance.

RESULTS

Characteristics of Healthy and GVHD Bone Marrow

Between September 2011 and January 2014, we obtained 12 bone marrow aspirates from patients with active acute or chronic GVHD who were being treated at either the Winship Cancer Institute, Emory University Hospital, or Children's Healthcare of Atlanta's Aflac Cancer and Blood Disorders Center (Table 1). Compared with marrow obtained from healthy volunteers, the marrow from GVHD patients had similar concentrations of leukocytes but had significantly lower platelet counts (Figure 1A) and a significantly higher frequency of monocytes (Figure 1B).

Growth Characteristics of Healthy and GVHD MSCs

We compared the growth kinetics of MSCs derived from bone marrow MNCs cultured in media supplemented with either phPL or FBS. Plating bone marrow MNCs in media supplemented with phPL resulted in more rapid and

Table 1
GVHD Patient Characteristics

Subject	Sex	Age (yr)	Malignancy Diagnosis	Date of Diagnosis	Date of Transplant	Type of Transplant	HLA Match	Post-Transplant Prophylaxis and Treatment	Date of GVHD Diagnosis	GVHD Type	Date of Marrow for MSC Isolation	Days from GVHD Diagnosis to Marrow
GVHD01	Female	55	HLL	1992	5/1/2011	Matched related donor: PBSC	10 of 10	Tacrolimus, methotrexate, prednisone	8/15/2011	Chronic overlap	9/20/2011	35
GVHD02	Female	14	Recurrent ALL	8/10/2007	11/22/2011	Matched related donor: marrow	8 of 8	Cyclosporine, methotrexate, topical hydrocortisone	12/21/2011	Acute grade I	1/18/2012	28
GVHD03	Female	9	ALL	5/13/2009	4/23/2012	Partially matched unrelated: cord	5 of 6	cyclosporine, mycophenolate, methylprednisolone, ATG, infliximab, budesonide	5/7/2012	Acute grade III	6/1/2012	25
GVHD04	Female	26	MDS	7/2/2012	10/22/2012	Matched related donor: PBSC	10 of 10	Tacrolimus, methotrexate, prednisone, methylprednisolone, infliximab, topical triamcinolone	12/14/2012	Acute grade III	2/1/2013	47
GVHD05	Male	50	AML	1/26/2012	9/14/2012	matched unrelated, donor: PBSC	10 of 10	Tacrolimus, methotrexate, prednisone	2/28/2013	Chronic overlap	3/14/2013	14
GVHD05	Female	2	JMML	10/18/2012	1/30/2013	Matched unrelated donor: marrow	8 of 8	Cyclosporine, methotrexate, methylprednisolone, orapred	2/18/2013	Acute grade III	3/19/2013	29
GVHD07	Female	29	AML	7/6/2011	3/29/2012	Matched unrelated, donor: PBSC	10 of 10	Tacrolimus, methotrexate, prednisone	1/9/2013	Chronic overlap	3/27/2013	77
GVHD08	Female	7	Pre-B ALL	8/23/2012	3/18/2013	Partially matched unrelated: cord	5 of 6	Cyclosporine, mycophenolate, methylprednisolone, budesonide, equine ATG, orapred	4/3/2013	Acute grade III	5/10/2013	37
GVHD09	Female	3	Pre-B ALL	10/12/2012	3/19/2013	Partially matched unrelated: cord	5 of 6	Cyclosporine, mycophenolate, methylprednisolone, equine ATG	4/2/2013	Acute grade IV	5/20/2013	48
GVHD10	Male	7	AML	5/14/2012	8/5/2013	Partially matched unrelated: cord	4 of 6	Cyclosporine, mycophenolate, methylprednisolone, prednisone	8/29/2013	Acute grade II	10/2/2013	34
GVHD11	Female	11	Pre-B ALL	3/15/2004	8/19/2013	Matched unrelated cord	6 of 6	Cyclosporine, mycophenolate, methylprednisolone, prednisone	9/13/2013	Acute grade II	10/18/2013	35
GVHD12	Male	8	T cell ALL	4/3/2013	12/11/2013	Partially matched unrelated: marrow	7 of 8	Cyclosporine, methotrexate, methylprednisolone, ATG, budesonide	12/26/2013	Acute grade III	2/4/2014	40

HLL indicates Hodgkin's lymphoma; PBSC, peripheral blood stem cell; ALL, acute lymphocytic leukemia; ATG, antithymocyte globulin; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; JMML, juvenile myelomonocytic leukemia.

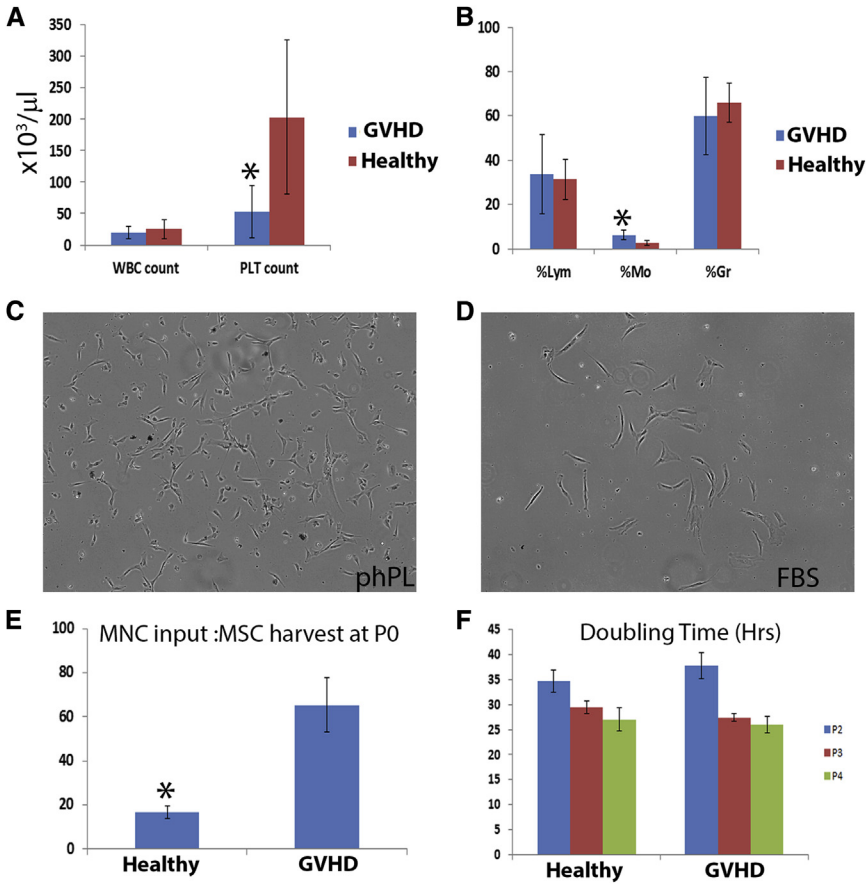

Figure 1. Isolation of MSCs from GVHD patients. (A and B) Comparison of marrow from GVHD and healthy subjects. (C and D) GVHD MSC colonies when expanded in phPL or FBS. (E) Isolation efficiency of MSCs from healthy and GVHD subjects. (F) MSC doubling time at passage 2-4 for healthy and GVHD MSCs. *$P < .05$, n = 11-12 individual MSC populations.

consistent MSC expansion (Figure 1C) compared with media supplemented with FBS (Figure 1D). In 4 GVHD MSC isolations the recovery and expansion of MSCs using FBS were directly compared with recovery and expansion of MSCs with phPL. MSC expansion occurred in 2 of the 4 GVHD marrow samples grown in FBS versus all 4 GVHD marrow samples grown in media supplemented with phPL. For the remaining 8 GVHD marrow samples, we solely expanded the cells in media with phPL and successfully isolated MSCs from all 8 samples. In the remainder of this work, we only compared GVHD MSCs and healthy MSCs expanded in media supplemented with phPL.

In comparing the initial recovery and expansion of MSCs from the bone marrow MNCs plated, we noted that marrow from healthy donors behaved in accordance with what we have seen historically. Typically, recovery of 1 MSC required plating an average (±SD) of 16.68 ± 2.92 MNCs for 9.5 ± 1.98 days (Figure 1E). Conversely, for a similar expansion period (9.36 ± 1.34 days), the number of GVHD MNCs that needed to be plated to recover 1 MSC was approximately 4 times (65.32 ± 12.36) that of healthy donors (Figure 1E). Despite this initial lag, once GVHD MSCs began proliferating ex vivo, their doubling times were similar to that of healthy donor MSCs (Figure 1F) [22].

Evaluating MSC Source

Although literature reports indicate that most MSCs derived from allo-HSCT patients are recipient derived [26-28], in several cases a small proportion of MSCs were reported to be of donor origin [29,30]. Because the prevalence of donor-derived MSCs in GVHD patients has not been determined, we used STR chimerism testing to determine the source of our GVHD MSCs. We evaluated 11 of the GVHD MSC populations we isolated and compared their STR signatures with that of the graft they received and the recipient's blood before transplant. In all 11 cases we found that the MSCs we expanded were exclusively recipient-derived (data not shown).

Immunophenotype, Differentiation, and Immunosuppressive Potential of Healthy and GVHD MSCs

As part of International Society for Cell Therapy guidelines to establish the identity of an MSC population [31], we cultured cryopreserved low passage (P3-4) MSCs from healthy and GVHD subjects for 1 week and analyzed their cell surface expressions of typical markers used to define MSCs. In our analysis of MSCs from 12 GVHD patients, we found that all 12 MSC populations isolated displayed a typical MSC phenotype with >95% of cells positive for CD44, CD73, CD105, CD90, and HLA-I and <5% of cells were positive for CD34, CD45, and CD19. To evaluate differences in the cell surface expression levels of markers between healthy and GVHD MSCs, we performed immunophenotyping of 3 healthy and 3 GVHD MSCs populations and calculated the mean fluorescent intensity for each marker. Despite some individual variations, there were no significant differences in

the overall mean fluorescent intensity of any cell surface markers analyzed between GVHD and healthy MSCs (Figure 2A). MSCs from 3 healthy donors and 3 GVHD patients were also cultured for 21 days under osteoblastic and adipogenic differentiation conditions and showed similar capacity to differentiated into mesodermal lineages (data not shown).

Immunomodulatory Effect of Healthy and GVHD MSCs

To evaluate the ability of GVHD MSCs to suppress T cell proliferation, third-party PBMCs were cultured for 4 days with either healthy or GVHD MSCs with or without anti-CD3/CD28 costimulation. In unstimulated PBMCs neither CD4 (7.66%) nor CD8 (6.73%) T cells proliferated appreciably, and baseline levels of proliferation were not altered by coculture with MSC. Conversely, stimulation of PBMCs alone with anti-CD3/CD28 beads caused both CD4 and CD8 T cells to proliferate extensively (72.5% and 67%, respectively), and coculture with MSCs could suppress this proliferation (Figure 2B). Coculturing either GVHD or healthy MSCs at different ratios with PBMCs (MSC/PBMC 1:5, 1:10, 1:20) demonstrated that healthy and GVHD MSCs equally and dose dependently suppressed T cell proliferation in vitro (Figure 2C). As a final measure of the immunomodulatory action of GVHD MSCs, we compared IDO gene expression after IFN-γ priming. MSCs were plated overnight and stimulated with 10 ng/mL IFN-γ for 4 hours or left untreated. After 4 hours MSCs were washed, lysed, RNA extracted, and reverse transcribed. The level of IDO expression in untreated MSCs was negligible, whereas 4-hour IFN-γ priming substantially increased IDO gene expression in both healthy MSCs and GVHD MSCs (Figure 2D).

DISCUSSION

In this study we tested whether MSCs derived from patients with GVHD are phenotypically and functionally analogous to MSCs derived from healthy donors. Our data demonstrate for the first time that despite an initial lag in expansion, clinical grade MSCs with potent immunomodulatory activity can be obtained from patients with active GVHD regardless of age, cancer diagnosis, or donor source. Consistent with several other studies [26-28], we also demonstrated that the MSCs derived from GVHD subjects were exclusively recipient derived.

The initial MSC growth defect we observed in GVHD samples is consistent with other published reports showing growth defects in MSCs derived from patients with several hematological malignancies, including acute lymphoblastic leukemia [19], acute myelogenous leukemia [20], and multiple myeloma [21] and is consistent with the concept that bone marrow stromal elements are susceptible to damage by both radiation and chemotherapy [19,20,32-36]. These studies would suggest that after bone marrow transplantation the

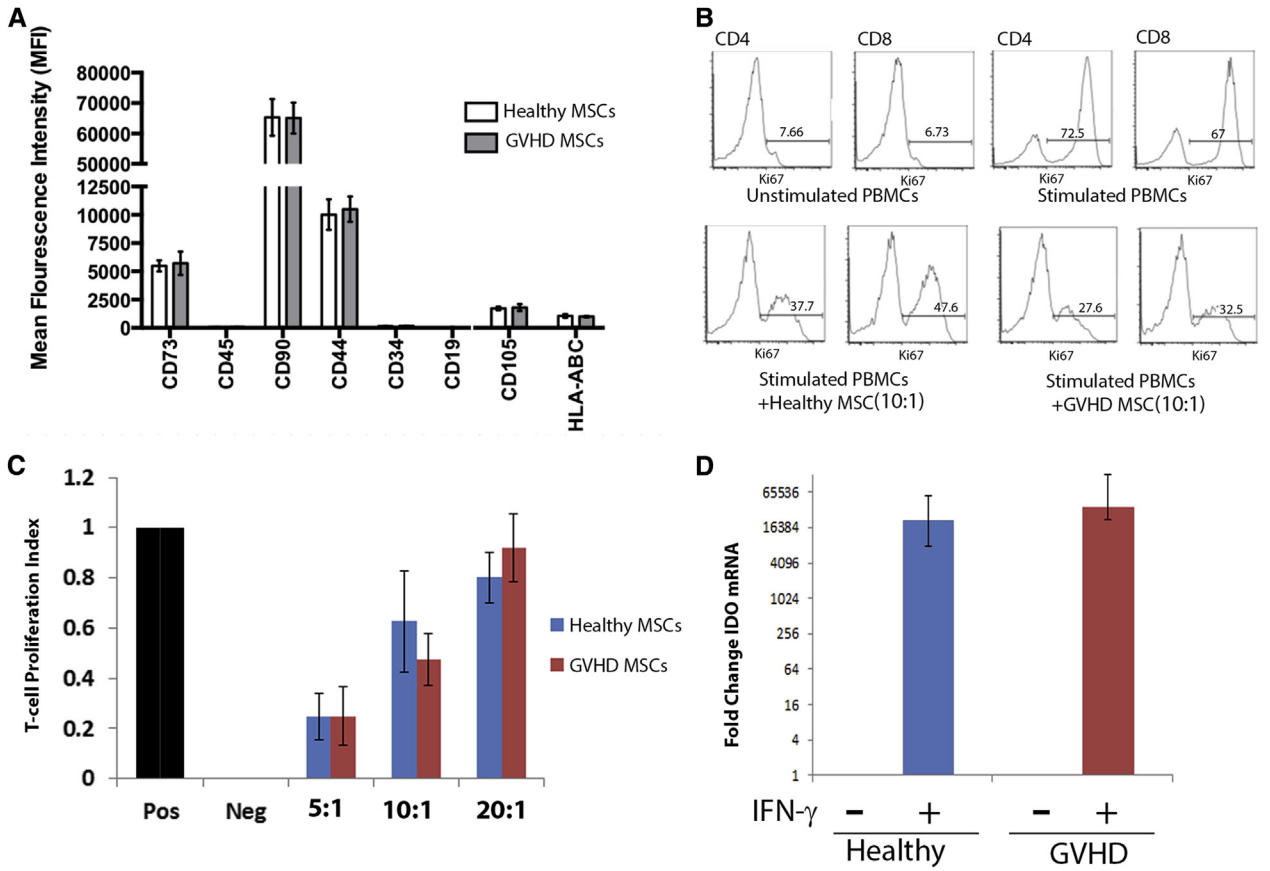

Figure 2. Identity and functional analysis of MSCs from GVHD patients. (A) Comparison of healthy and GVHD MSCs immunophenotype measured by flow cytometry. (B) Representative flow plots of T cell proliferation assays performed using PBMCs cultured for 4 days unstimulated, activated with anti-CD3/CD28 Dynabeads, or activated with anti-CD3/CD28 Dynabeads and cocultured (10:1 ratio) with either healthy or GVHD MSCs. (C) T cell proliferation suppression index of PBMCs anti-CD3/CD28 stimulated (Pos) or cocultured for 4 days with varying ratios of MSC:PBMC with either healthy or GVHD MSCs. (D) Relative IDO gene expression analyzed by real-time PCR of healthy or GVHD MSC without or pretreated with 10 ng/mL of recombinant human IFN-γ for 4 hours. n = 3-5 individual MSC populations.

clinical utility of the recipients' MSCs are negligible. Our data dispute this premise because we successfully isolated and expanded MSCs from 3 subjects with acute myelogenous leukemia and 6 with acute lymphoblastic leukemia. In all cases we found that after the initial lag phase, GVHD MSCs proliferated at similar rates to those of our healthy volunteers. One possible explanation for this discrepancy could be our utilization of phPL to expand our MSCs as opposed to FBS, which others have typically used. Along with ourselves, numerous investigators have shown that phPL is superior to FBS in expanding MSCs [22,37], and recently we demonstrated that phPL can rejuvenate presenescent MSCs [38]. Thus, we postulate that phPL for the ex vivo expansion of GVHD MSCs can counteract the negative effects conditioning regimes have on MSCs and may revitalize GVHD MSCs from a presenescent-like MSC phenotype as has been described for MSCs from multiple myeloma patients [21].

Regardless of whether or not MSCs can be isolated from GVHD subjects, if the resulting MSCs are not functionally competent, their clinical utility is dubious. Functional analyses by others suggest that the toxic conditioning used in allo-HSCT impairs the ability of MSCs to support HSC maintenance and growth [20,39-42]; however, whether these same conditioning regimes compromise the ability of MSCs to be immunosuppressive has not been ascertained. Several studies have shown that MSCs derived from multiple myeloma patients have impaired immunomodulatory activity [32,43,44]; however, our data with non–multiple myeloma subjects suggests that GVHD MSCs can actively suppress T cell proliferation and can do so via tryptophan catabolism through the IDO pathway [45]. From these observations we conclude that the conditioning regimes and maintenance regimes used in our GVHD population did not impact the ability of their MSCs to be immunosuppressive. Supporting this concept, Wobus et al. [46] exposed MSCs from healthy donors to lenalidomide and found that lenalidomide impaired the capacity of MSCs to direct migration of CD34(+) HSCs but did not affect their immunomodulatory ability.

Our data support the idea that autologous GVHD MSCs (like healthy allogeneic MSCs) can induce tolerance by reducing the expansion of autoreactive T cells and by inducing regulatory T cells [47,48], but unlike allogeneic MSCs, autologous GVHD MSCs may also facilitate durable tolerance by inducing anergy. Anergy induced tolerance in solid organ transplant is based on the premise that bolus infusion of donor cells mediates the elimination of donor-specific recipient effector T cells but leaves the remainder of the T cell repertoire intact [49-51]. In contrast to solid organ transplant rejection, GVHD is driven by donor lymphocytes against host antigens, meaning bolus infusion(s) of recipient cells may help to induce anergy. Supporting this concept are data from a murine nonmyeloablative allogeneic bone marrow transplant study that showed that autologous MSCs infusion enhanced long-term engraftment and tolerance [17], whereas infusion of allogeneic MSCs lead graft failure. Data by Florek et al. [52] also showed that infusion of apoptotic autologous splenocytes 24 hours before conditioning could delay the onset of GVHD in a murine model. Whether autologous human MSCs for GVHD can induce anergy is unproven; however, it does offer an attractive secondary mechanism by which tolerance may be established and can be tested clinically.

Based on these data we conclude that recipient-derived MSCs from patients with GVHD are analogous to MSCs from healthy volunteers and represent a viable option for clinical testing as an immunomodulatory option for symptomatic GVHD.

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